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# A New Vector System with Inducible E2a Cell Line for Production of Higher Titer and Safer Adenoviral Vectors

Heshan Zhou*,†,‡ and Arthur L. Beaudet*,†,[1]

*Department of Molecular and Human Genetics, †Department of Pediatrics, and ‡Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas 77030

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Adenoviral vectors have been used in gene therapy and for vaccination. The major concerns with using adenoviral vectors are the pathogenic potential of the virus backbone and the generation of replication-competent adenovirus that may replicate in an uncontrolled manner, especially in immunocompromised patients. It is important to develop new vectors that are safer for clinical trials while maintaining high titer and efficient transduction. A new adenovirus vector production system was developed, which includes several vector backbone plasmids deleted for E2a and a new cell line expressing both E1 and E2a. The new cell line with the tTA-inducible E2a expression cassette can significantly increase the titer of E1/E2a-deleted vectors by four to five orders of magnitude upon withdrawal of tetracycline. Furthermore, there is no sequence overlap between the vector and the cellular DNA within the E2a open reading frame and downstream, making the generation of virus with wild-type E2a through homologous recombination substantially less likely. The new vector system may improve the safety of vectors for vaccination and cancer therapy and may also provide safer backbones for further vector development, such as helper-dependent and hybrid vectors. 

## INTRODUCTION

Human adenoviral (Ad) vectors are one of the more efficient vehicles for transferring foreign genes into mammalian cells for vaccination and for gene therapy of cancer and hereditary diseases. Ad vectors have been well characterized, are easy to manipulate, and can be grown to high titers. One potential advantage of Ad recombinant vaccines is their ability to elicit protective cell-mediated immunity and humoral responses to the antigen delivered by the vector (Randrianarison-Jewtoukoff and Perricaudet, 1995). The vectors can also induce mucosal immune responses that are particularly important in AIDS vaccine development. For example, Ad vectors carrying the HIV-1 gp120 gene or the simian immunodeficiency virus pg120 gene resulted in high-titer neutralizing antibodies and cellular and mucosal immune responses; this provided long-term protection following AIDS virus challenge (Lubeck *et al.,* 1997; Buge *et al.,* 1997). One additional application of Ad vectors is their use in cancer gene therapy (Zhang, 1999). The approaches include induction of anticancer immunity through the delivery of either immunomodulatory genes, such as interleukin-12 (Davidoff *et al.,* 1999), or the genes of tumor-specific antigens, such as polyoma middle T (Wan *et al.,* 1999).

The major concern with using recombinant Ad vectors is the pathogenic potential of the adenovirus backbone. Most Ad vectors used in vaccines are E1-substituted first-generation vectors (Randrianarison-Jewtoukoff and Perricaudet, 1995; Zhang, 1999). The E1-substituted vectors are usually produced in 293 cells generated by the transfection of human kidney cells with sheared Ad serotype 5 (Ad5) DNA (Graham *et al.,* 1977). Mapping of the Ad sequences in the 293 cell line has indicated the presence of contiguous Ad5 sequences from the left-hand end of the genome up to position 4137 (Louis *et al.,* 1997). When typical E1-substituted vectors, which contain a deletion of the E1 region from 340 to 3500 of the Ad genome, are propagated in 293 cells, sequence overlap frequently results in the generation of replication-competent adenovirus (RCA) (Lochmuller *et al.,* 1994; Hehir *et al.,* 1996; Fallaux *et al.,* 1999). RCA has the potential to replicate in an uncontrolled manner in patients, especially in immunocompromised individuals such as AIDS or cancer patients. The presence of RCA in Ad vector preparations for clinical use may cause complications and is clearly undesirable. One approach to prevent RCA is the development of cell lines lacking overlapping sequences (Fallaux *et al.,* 1998; Gao *et al.,* 2000).

The other concern with E1-subtituted Ad vectors is that the vectors may still be able to replicate even with the

[1] To whom correspondence and reprint requests should be addressed at Baylor College of Medicine, Department of Molecular and Human Genetics, Room T619, Houston, TX 77030. Fax: (713) 798-7773. E-mail: abeaudet@bcm.tmc.edu.

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deleted E1 region. Research has shown that virus with a mutated E1 region did not completely eradicate the synthesis of viral proteins and DNA (Winberg and Shenk, 1984; Shenk and Williams, 1984). The synthesis of the viral DNA and proteins has been observed when E1-deleted vectors were used to infect noncomplementing cells (Mittereder *et al.,* 1994). Host cellular factors may provide the "E1-like" function for replication of E1-deleted vectors (Imperiale *et al.,* 1984). In addition, adenovirus can replicate very well, even if extremely low levels of E1 are expressed (Hearing and Shenk, 1983; Hitt and Graham, 1990). The 293 cell line, containing a single copy of the E1 region (Louis *et al.,* 1997), can propagate Ad vectors to titers of 10,000 or more per cell. This further suggests that small amounts of E1 or E1-like factors could be sufficient to allow plentiful amplification of Ad vectors. Hence, Ad vectors with E1 deletion may cause unpredictable outcomes in clinical trials. It is important both to achieve optimal expression of the therapeutic gene and to avoid uncontrolled synthesis of Ad vectors in therapy. One strategy is to develop new vectors with additional deletions of other essential viral genes. Research demonstrated a long time ago that an adenovirus with deletion of another gene (E2a) could replicate in complementing cells (Klessig *et al.,* 1984).

The so-called second-generation vectors with deleted E1/E2 or E1/E4 have been developed and the vectors can be propagated in cell lines that complement both E1 and E2 or E1 and E4 (Zhou *et al.,* 1996; Gorziglia *et al.,* 1996; Gao *et al.,* 1996; Amalfitano *et al.,* 1998; Lusky *et al.,* 1998). Vectors with E1/E2 deletions cannot replicate in noncomplementing cells and therefore may improve safety in gene therapy (Zhou *et al.,* 1996; Lusky *et al.,* 1998; Gorziglia *et al.,* 1996). However, the advantages of the vectors with regard to extending transgene expression remain to be studied further (Amalfitano, 1999). The E1/E2-deleted vectors have resulted in immune responses similar to those elicited by the vectors with only the E1 region deleted, as we have previously demonstrated (Morral *et al.,* 1997). This may limit the vector application in gene therapy for hereditary diseases, but should be attractive for the development of viral recombinant vaccines. To further improve the E1/E2a-deleted vector systems, we have developed several vector backbone plasmids deleted for E2a open reading frame (ORF) and a new inducible E2a complementing cell line, E2T. There is no sequence overlap between the vector and the cellular DNA within the E2a ORF and downstream. The titer of E1/E2a-deleted vector was significantly increased in response to withdrawal of tetracycline and no E2a wild-type virus could be detected in the vector preparations. Construction and preparation of the E1- and E2a-deleted vectors has been simplified. The new vector system may be especially useful for vaccine and cancer therapy.

## RESULTS

### Development of inducible E2a-complementing cell lines

Because high-level expression of the E2a product is essential for preparation of high-titer Ad vectors with deletion of E2a, the tetracycline-regulated system (Gossen and Bujard, 1992; Resnitzky *et al.,* 1994) was used for development of E2a-expressing inducible cell lines. This system has two components. The transactivator protein tTA, encoded in plasmid pUHD15-1, is a fusion protein composed of the repressor of the tetracycline operon (*tet*R) from *Escherichia coli* (Tn 10) and the activating domain of herpes simplex virus protein 16 (Gossen and Bujard, 1992). The PhCMV*-1 region, in the response plasmid pUHD10-3, contains the CMV core promoter and several repeats of *tet* operator (*tet*O) sequences (Resnitzky *et al.,* 1994). In this system, the tTA binds to the *tet*O and induces transcription from the PhCMV*-1 promoter in the absence of tetracycline, but not in its presence. We modified the system for facilitation of development of the E2a-complementing cell line. First, a selective marker neomycin-resistant gene was introduced into the regulator plasmid pUDH15-1 at the unique *Xho*I site, resulting in a new plasmid designated pBZ47 (Fig. 1). Second, the CMV core promoter was deleted from the PhCMV*-1 region in plasmid pUHD10-3, but the *tet*O sequences were retained. The E2a ORF, with its endogenous early promoter (E-P) and late promoter (L-P) cloned from pFG140, was inserted into the modified pUHD10-3. In the final plasmid construct, pBZ40, the E2a E-P and L-P were linked to the *tet*O, so that the tTA transactivator could enhance the E2a expression (Fig. 1). No sequences downstream from the stop codon of the E2a ORF were included in the construct.

After cotransfection with the pBZ40 and pBZ47 plasmids into 293 cells, over 100 G418-resistant colonies were selected. Only 1 of the colonies (293-G7) had efficient inducible complementing ability upon withdrawal of tetracycline from the culture medium (Fig. 2). Unfortunately, the inducible 293-G7 cell line did not produced a high titer of E2a-deleted vector even compared with the previous noninducible 293-C2 cell line (Fig. 2) (Zhou *et al.,* 1996). We reasoned that introducing multiple copies of the E2a-expressing cassette into 293-G7 cells could increase the E2a-complementing ability. We did a second cotransfection of the E2a-expressing plasmid pBZ40 into 293-G7 cells with another selective plasmid, pPGK-purobpA, containing a puromycin resistance cassette (provided by Dr. Allan Bradley). Two resistant colonies (293E2T-1 and 293E2T-11) that could significantly increase the titer of the E2a-deleted vector in culture upon withdrawal of tetracycline were selected from puromycin-resistant clones.

To compare the titers of an E2a-deleted vector with a comparable vector containing a wild-type (WT) E2a re-

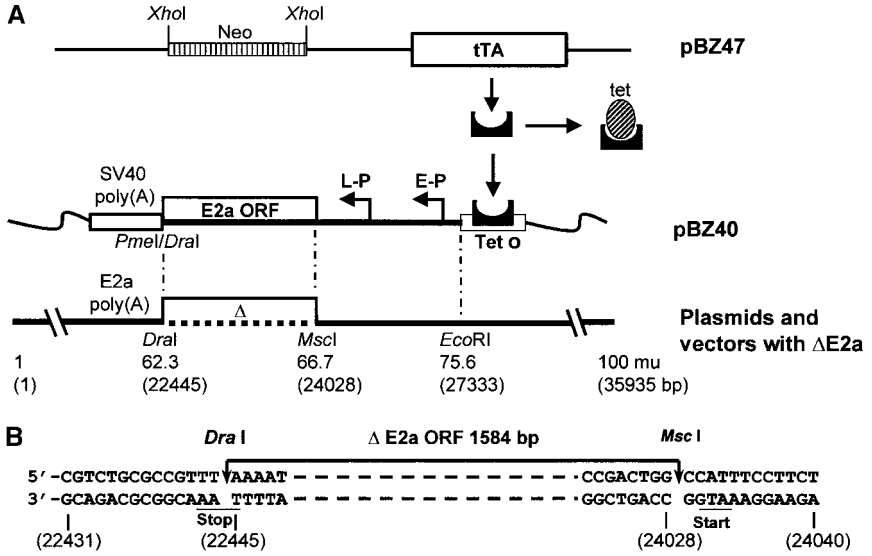


FIG. 1. Depiction of the DNA segments in plasmids used to develop the inducible E2a-complementing cell lines and to construct vectors with deletion of E2a. (A) The tTA gene and neomycin resistance cassette in plasmid pBZ47 are shown at the top. Plasmid pBZ40 containing the adenoviral fragment and tetracycline operator sites (*tet*O) is in the middle. Transactivator protein tTA interacts with *tet*O, enhancing transcription from E2a promoters when tetracycline is absent. The addition of tetracycline inhibits transcription. The structure of plasmids and vectors deleted for E2a from bp 22445 to 24028 is shown. (B) The restriction enzyme sites used to delete the E2a ORF and the sequences around the deleted sites in plasmids and adenoviral vectors are shown. The start and stop codons of E2a gene are depicted. All of the map units (mu) and nucleotide sequence coordinates refer to Ad5 (GenBank Accession No. M73260).

gion, we used our previously constructed Ad$\beta$galΔE1E2 (deleted E2a) and Ad$\beta$galΔE1 (WT E2a) vectors (Zhou *et al.,* 1996) to infect 293 and different E2a-complementing cells at a multiplicity of infection (m.o.i.) of 1. The cells were cultured in medium with or without tetracycline. After 2 days of infection, the total cultures with cells were collected; lysates were prepared and serially diluted to determine the titers. The titers of the Ad$\beta$galΔE1 containing WT E2a were the same regardless of cell lines and tetracycline, because the Ad$\beta$galΔE1 did not depend on E2a complementation from cells. The titers of Ad$\beta$galΔE1E2 vector in noninducible 293-C2 cells (Zhou *et al.,* 1996) were relatively low compared to the E2a-WT vector and there was no difference whether adding tetracycline in medium or not. However, 293E2T-1 and 293E2T-11 cells increased the titer of the E2a-deleted vector four to five orders of magnitude upon withdrawal of tetracycline in cultures, with the titer reaching levels similar to those of the E2a wild-type vector (Fig. 2). The E2a-deleted vector could not replicate in 293 cells.

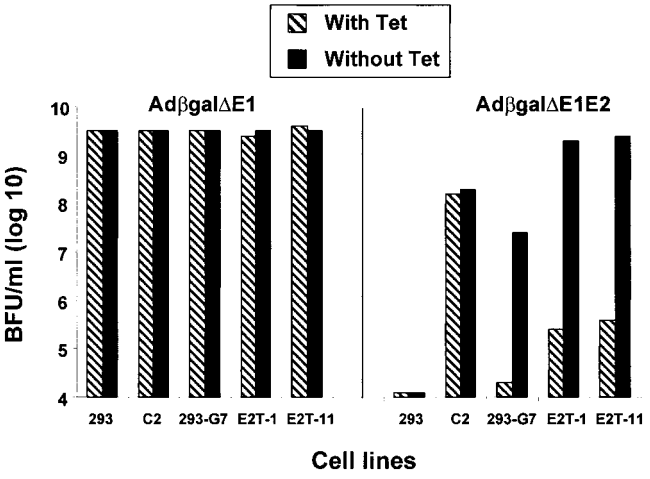


FIG. 2. Comparison of the titers of viruses with wild-type E2a or with deletion of E2a on 293 cells and E2a-complementing cells. Titer of the virus with wild-type E2a, Ad$\beta$galΔE1, is shown on the left, and titer of the virus with E2a deletion, Ad$\beta$galΔE1E2, is shown on the right. Hatched bars indicate viruses produced in medium containing 10 $\mu$g/ml tetracycline, and solid bars indicate viruses produced in a medium without tetracycline. Virus was collected from the medium after 2 days of infection with an m.o.i. of 1, and the titer was measured as blue forming units (BFU) on E2T cells. The titer of Ad$\beta$galΔE1E2 in 293 cells is not higher than can be accounted for by the input virus, because the E2a-deleted virus cannot replicate (Zhou *et al.,* 1996). Ad$\beta$galΔE1E2 can replicate in uninduced E2T cells (with tet) with titers substantially below those with induced E2T cells (without tet).

## Multiple copies of E2a in complementing cells

It was possible that the higher yield of the E2a-deleted vector in the 293E2T-1 and 293E2T-11 cells, compared to 293-G7 cells, was due to multiple E2a cassettes in these cells. To evaluate this, the copy number of the E2a gene was determined by Southern hybridization. About 20 copies of the E2a gene were present in both 293E2T-1 and 293E2T-11 cells, while there were only 1 or 2 copies in 293-G7 and 293-C2 cells (Fig. 3). Since there was no difference between the 293E2T-1 and the 293E2T-11 cell lines, we focused on the 293E2T-1 (E2T) cells in subsequent studies.

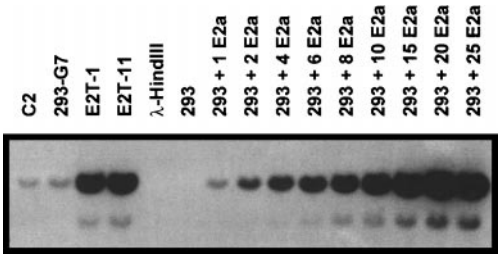


FIG. 3. Southern blot analysis of the E2a copy number in C2, G7 293E2T-1, and 293E2T-11 genomic DNA. The probe was a 1.75-kb PCR product amplified from the E2a coding region of AdFG140 DNA with primers of 731 and 732 (Fig. 7). Standard lanes contain 1 to 25 copies of viral E2a, each mixed with 10 $\mu$g of 293 genomic DNA. The DNA samples were cleaved with restriction endonucleases *Eco*RI and *Dra*I.

### E2a protein induced in the E2T cell upon withdrawal of tetracycline

Expression of E2a was analyzed by Western blotting. As a positive control, 293 cells were infected with the E2a WT vector AdFG140; as a negative control, 293 cells were infected with an E2a-deleted vector AdFG140ΔE2. E2T cells were grown in medium with or without tetracycline. Cell lysates were isolated from these cells for Western blotting. As expected, 293 cells infected with the E2a WT vector had a high level of E2a expression from the vector, in contrast to undetectable E2a in 293 cells infected with the E2a-deleted vector. The level of the E2a gene product in E2T cells cultured in medium without tetracycline was about threefold higher than that from the same cells cultured in medium containing tetracycline (Fig. 4). This provides direct evidence that the transcriptional activity of the E2a endogenous promoters in the E2T cells was enhanced by the tetracycline system. In the noninduced condition (with tetracycline), the protein was still produced from the cell because of the basal activity of the endogenous E2a promoters. To test whether infection of E2a-deleted adenovirus would inhibit expression of E2a in the complementing cells, we infected the E2T cells with AdFG140ΔE2. The results showed that infection with the E2a-deleted vector did not inhibit expression of E2a in the E2T cells under both induced and noninduced conditions (Fig. 4).

### Inducing the titer of E2a-deleted adenovirus with tetracycline

E2T cells were grown under the same conditions as 293 cells, except for the addition of 10 $\mu$g tetracycline/ml to keep the E2a expression at relatively lower levels. There was no detectable difference between E2T and 293 in growth characteristics and morphologies after the E2T cell cultured for several years and over at least 20 passages (Fig. 5). When E2T cells were infected with Ad$\beta$galΔE1E2 at an m.o.i. of 0.1, complete CPE was only observed in 2 days in the E2T culture without tetracycline, while partial CPE was observed in the culture with tetracycline. The E2a-deleted vector did not cause 293 CPE (Fig. 5).

The E2a-deleted Ad$\beta$galΔE1E2 was also used to infect E2T cells cultured in medium with various concentrations of tetracycline. The titer of the vector produced in cell culture was induced from about $10^5$ to $10^9$ blue forming units (BFU)/ml, while the tetracycline concentration was decreased from 10 to 0 $\mu$g/ml. The production of the E2a-deleted vector was inhibited at levels as low as 0.1 $\mu$g/ml of tetracycline (Fig. 6). The above experiments indicated that decreasing concentrations of tetracycline in medium resulted in higher yields of E2a-deleted vector.

### Development of adenoviral vectors with deletion of the entire of E2a ORF

To substantially reduce the possibility of homologous recombination in the E2a sequence between the E2T cells and the vectors, we deleted a segment of 1584 bp covering the entire E2a ORF from Ad genomic plasmids pFG140, pBHG10, and pBHE3 to generate plasmids pBZ66, pBZ68, and pBZ71, respectively (Fig. 1 and Table 1). Multiple restriction sites (*Pac*I–*Xba*I–*Bst*B1–*Cla*I) were introduced into the deleted region to allow cloning transgenes in the deleted E2a region. With the previously established method for generating first-generation Ad vectors (Bett *et al.,* 1994), the E2a-deleted plasmid pBZ66 could be directly converted into an E2a-deleted Ad vector (AdBZ66ΔE2) in the E1- and E2a-complementing E2T cells (Table 1). Plasmids pBZ68 and pBZ71 have been used to rescue second-generation vectors with multiple deletions of E1/E2a/E3 or E1/E2a (data not shown). All of these plasmids and Ad vectors were documented to contain the appropriate deletions as exemplified in Fig. 7 by PCR analysis of AdBZ66ΔE2. The deletion of the E2a

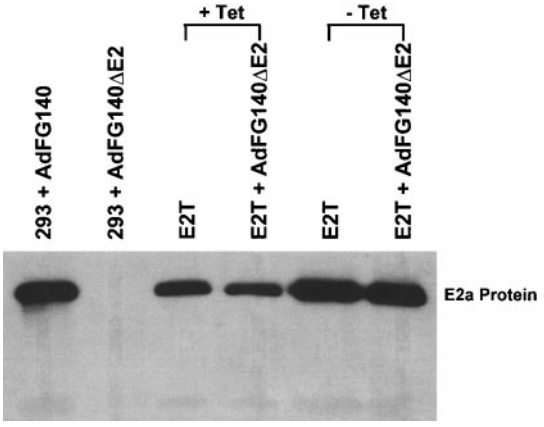


FIG. 4. Western analysis of E2a protein produced in E2T cells. The E2a gene product DBP was detected by Western blot as described under Materials and Methods. DBP was seen in 293 cells infected by AdFG140 with wild-type E2a, but not in 293 cells infected with E2a-deleted AdFG140ΔE2. E2T cells expressed increased levels of DBP when tetracycline was omitted from the medium.

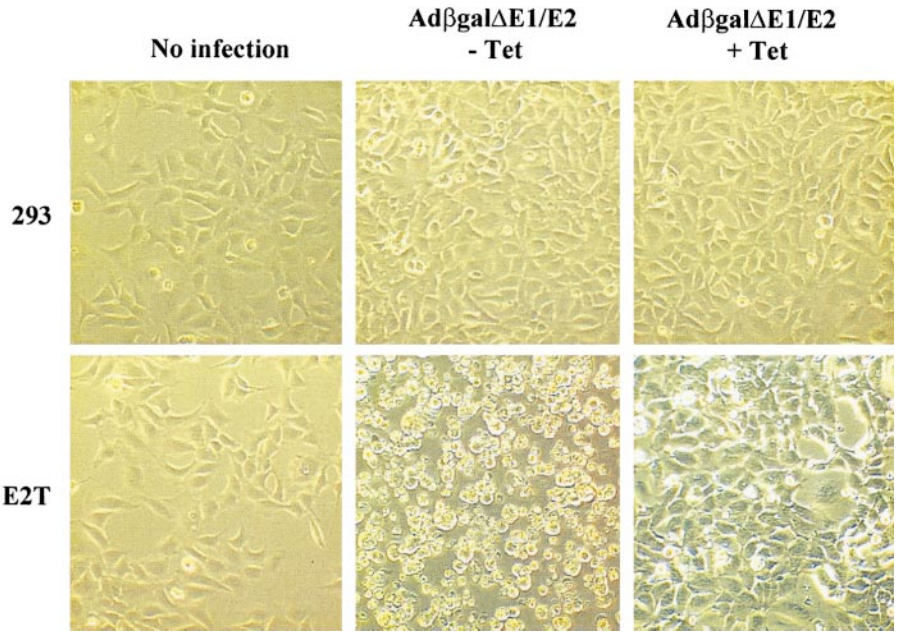


FIG. 5. Comparison of 293 cells and E2T cells with regard to morphology and CPE. The 293 cells and E2T cells were uninfected (left) or infected with E2a-deleted vector at an m.o.i. of 0.1 without tetracycline (middle) or with tetracycline (right) in medium.

gene in these vectors was confirmed by restriction enzyme analysis with *Hin*dIII, *Asc*I, and *Eco*RI–*Bam*HI (data not shown).

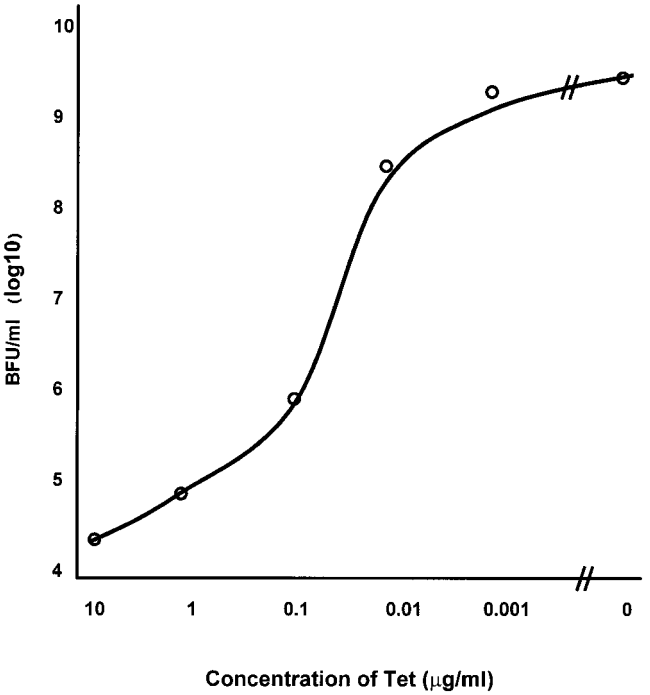


FIG. 6. Growth of E2a-deleted vector in E2T cells with different concentrations of tetracycline in medium. Ad$\beta$galΔE1E2 was used to infect E2T cells cultured in medium with different concentrations of tetracycline. The vector titer increased with decreasing concentrations of tetracycline.

## Absence of detectable E2a wild-type virus from preparations of second-generation Ad vectors

To test whether E2a WT virus would emerge during amplification of the E2a-deleted vectors in E2T cells, we successively infected E2T cells with the AdBZ66ΔE2 virus. After a total of 20 serial passages, the virus was assayed for WT E2a. AdBZ66ΔE2 was compared with AdFG140 containing a WT E2a region prepared from 293 cells and AdFG140ΔE2 containing partially deleted E2a prepared from the previous E2a-complementing 293-C2 cells (Zhou *et al.,* 1996). PCR with a pair of primers, one (551) located within the deleted E2a ORF and another (731) located outside of the ORF, was specifically used to assess the presence of the WT E2a sequence. With primers 551 and 731, a fragment of about 0.68 kb representing the WT E2a PCR product was amplified from E2a-WT AdFG140. For the E2a partial-deleted AdFG140ΔE2 which contains overlapping sequences with the complementing 293-C2 cells, there was a weak band of a 0.68-kb fragment when primers 551 and 731 were used (Fig. 7). This suggested the emergence of E2a WT virus from AdFG140ΔE2 vector propagated in 293-C2 cells. However, this 0.68-kb fragment could not be detected for AdBZ66ΔE2 propagated in the new E2T cells (Fig. 7). Primers 731 and 732 flanking the E2a ORF yielded a 1.75-kb fragment for AdFG140 and shortened fragments for AdFG140ΔE2 and AdBZ66ΔE2 (Fig. 7). PCR with primers of 731 and 732 did not yield the WT 1.75-kb fragment for AdFG140ΔE2, because when there was only a small amount of WT E2a viruses, the primers favor amplification of the shorter (0.48-kb) fragment. In the past

TABLE 1

Plasmids and Viral Vectors

| Plasmid or vector | Description | Reference |
|---|---|---|
| Plasmids | | |
| pFG140 | Insertion of 2.21 kb in E1; dl309 in E3 | Graham, 1984 |
| pBHGE3 | Deletion of packaging signal and E1 | Bett *et al.,* 1994 |
| pBHG10 | Deletion of packaging signal, E1, and E3 | Bett *et al.,* 1994 |
| pΔE1sp1A | Shuttle for adenovirus vector | Bett *et al.,* 1994 |
| pUHD10-3 | Response plasmid in tTA-dependent expression system | Resnitzky *et al.,* 1994 |
| pUHD15-1 | Contains transactivator protein tTA | Gossen and Bujard, 1992 |
| pBZ40 | Inducible E2a-expressing plasmid, derived from pUHD10-3 | This report |
| pBZ47 | Contains protein tTA and Neo-resistant, derived from pUHD15-1 | This report |
| pBZ66 | pFG140ΔE2a-ORF | This report |
| pBZ68 | pBHG10ΔE2a-ORF | This report |
| pBZ71 | pBHGE3ΔE2a-ORF | This report |
| pBZ92 | Shuttle containing β-gal flanked with ITR of AAV, derived from pΔE1sp1A | This report |
| pPGKpurobpA | Contains a puromycin resistance cassette | Provided by A. Bradley |
| Viral vectors | | |
| Adβgal ΔE1 | β-Gal-expressing vector with E1 deleted[a] | Zhou *et al.,* 1996 |
| Adβgal ΔE1E2 | β-Gal-expressing vector with E1 and E2 deleted | Zhou *et al.,* 1996 |
| AdFG140 | Vector derived from pFG140 | Zhou *et al.,* 1996 |
| AdFG140ΔE2 | Vector derived from pFG140ΔE2 | Zhou *et al.,* 1996 |
| AdBZ66ΔE2 | Vector derived from pBZ66 | This report |

[a] β-gal, β-galactosidase.

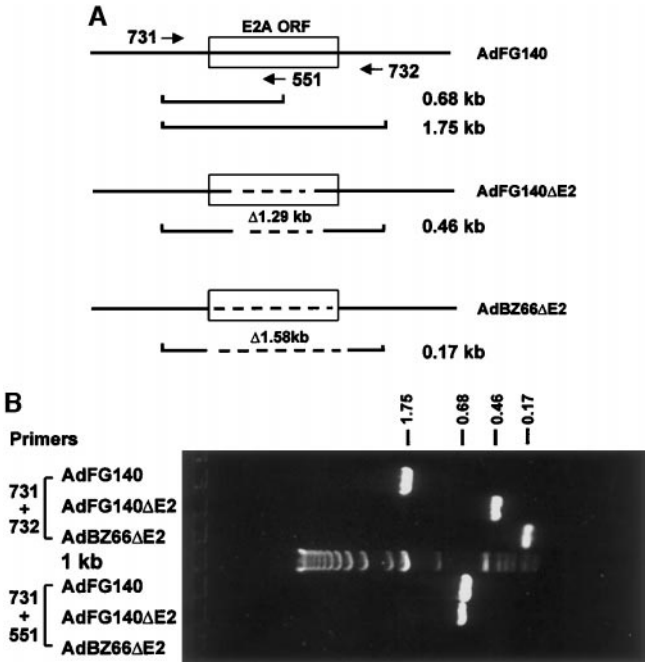


FIG. 7. PCR analysis of the deleted E2a region in adenoviral vectors. (A) Primers within and flanking the E2a ORF are indicated at the top. (B) Primers flanking the E2a ORF yielded full-length segments for AdFG140, which contains wild-type E2a, while the appropriate smaller products were seen for the vectors with partial or completely deleted E2a ORF. The primer within the E2a ORF yielded a 0.68-kb fragment for AdFG140 and AdFG140DE2 (partial deletion), but did not yield any PCR product for AdBZ66ΔE2 (deleted entire E2a).

few years we have used E2T cells to make multiple preparations of E2a-deleted vectors. No evidence of contamination with WT E2a was found in any of these preparations (O'Neal *et al.,* 1998).

## DISCUSSION

The E1-substituted first-generation Ad vectors have been used in gene therapy and vaccination (Randrianarison-Jewtoukoff and Perricaudet, 1995; Zhang, 1999). However, the potential pathogenicity of Ad vectors requires particular attention, especially in infants and in AIDS and cancer patients who are candidates for vaccine treatment. Emergence of RCA in final batches of E1-substituted vectors and possible preexisting E1-like factors in some host cells may cause uncontrolled adenoviral replication in humans. Ad vectors with additional deletions in other critical viral genes may benefit Ad-mediated vaccination and gene therapy. The DNA-binding protein (DBP) encoded by the Ad E2a gene is essential for viral DNA replication and for transcription from the major late promoter that controls the synthesis of all viral structural proteins (Shenk, 1996). Previous studies confirmed that, unlike the first-generation vectors, vectors with deletions in E1 and E2a do not express detectable adenovirus early and late proteins and do not synthesize viral DNA in noncomplementing cells (Gorziglia *et al.,* 1996; Christ *et al.,* 1997; Rittner *et al.,* 1997; Lusky *et al.,* 1998). Vectors with deletions of both E1 and E2a can efficiently deliver foreign genes into animals, but these vectors still elicited immune responses against the

transgene protein as did first-generation vectors (Zhou *et al.,* 1996; Morral *et al.,* 1997). Hence the E1/E2a-deleted vectors may have limited application in gene therapy for hereditary disease, but should be attractive for recombinant viral vaccines.

Using the previously described E1/E2a-deleted vector system, construction and preparation of the E1/E2a-deleted vector was complicated compared to the first-generation Ad vectors. The titer of E2a-deleted vectors was generally lower than that of the vectors with the E2a region intact, and there still was the possibility of the emergence of RCA due to the overlapping sequence in the E2a region between the vectors and the cell line. These factors have hindered the use of E2a-deleted vectors for vaccine applications in human clinical trials.

To increase the titer of E2a-deleted vector, we applied the tTA-inducible system to enhance E2a promoter activity in complementing cells. When E2T cells are cultured in medium containing tetracycline, the E2a expression is under the control of its endogenous promoters. Removing tetracycline from the medium increased E2a protein by threefold in the cells (Figs. 1 and 4). An important feature of the new cell line is that withdrawal of tetracycline from the medium can significantly increase the titer of E2a-deleted vector by four to five orders of magnitude (Figs. 2 and 6). It is not certain how a few-fold increase of E2a product results in a four to five orders of magnitude increase of the vector titer. Although adenovirus replication is directly dependent on the E2a product, this may not necessarily be a linear relationship. Under the induced condition, the E2a product in E2T cells increased to a level similar to that of 293 cells infected with the E2a WT vector (Fig. 4); this may be adequate for maximal virus production. Since high levels of E2a may be toxic to cells, we generally add tetracycline (final concentration, 10 $\mu$g/ml) in the maintenance cultures. Under this condition, the cell line is quite stable over the years even if the E2a is still expressed from its endogenous promoters. The E2T cells did not have any detectable morphological changes compared with 293 cells under the microscope (Fig. 5).

In this vector system, there are no homologous sequences between the vector DNA and the cellular DNA in the region immediately downstream of the E2a ORF. The E2a promoter region does exist in the E2T cell line and overlaps with the vectors (Fig. 1). However, with sequence overlap only at one end of the E2a ORF, there is a substantially reduced potential for a double-crossover event necessary for the generation of an E2a wild-type virus or vector. The vector system will provide a relatively safer gene delivery method for gene therapy and vaccination. This system reported here is based on the 293 cell line. Therefore, the possibility of generating E1 WT virus with an E2a deletion still exists. A further improvement of this system would be to develop cell lines that do not contain homologous sequences in the E1 region as in PER.C6 (Fallaux *et al.,* 1998) and GH329 (Gao *et al.,* 2000).

First-generation Ad vectors with E1 and E3 deletions can accommodate up to 8 kb of inserted DNA (Bett *et al.,* 1994). With the added deletion of 1.6 kb in the E2a ORF, the packaging capacity of Ad vectors increases to about 10 kb. The added capacity for an E1/E2a/E3-deleted Ad vector will permit the delivery of larger or multiple foreign genes or the delivery of complex inducible sequences, such as the tTA or RU486 (Wang *et al.,* 1994) systems, to regulate foreign gene expression. Another direction for vector development is to construct hybrid vectors, such as Ad-adeno-associated virus and Ad-retrovirus vectors, which may combine advantages from two different viruses. Our E2a-deleted vectors will not only expand the capacity, but also provide a safer backbone for such construction.

Recently developed helper-dependent (HD) Ad vectors can significantly decrease toxicity and immune responses and prolong transgene expression (Haecker *et al.,* 1996; Schiedner *et al.,* 1998; Morral *et al.,* 1998, 1999). In the HD-Ad vector system, a modified first-generation Ad vector is used as a helper virus. Since multiple coinfections of the helper virus and the HD-Ad vector are necessary to produce the HD-Ad vector, the contamination of helper virus in the HD-Ad vector preparations cannot be avoided with the current 293Cre cell lines. In addition, there is substantial possibility for the occurrence of RCA in the HD-Ad vector preparations (Parks *et al.,* 1996). Based on the E1/E2a-deleted vector system reported here, the E1/E2a-complementing cell line and the E1/E2a double-deleted vector have been modified for preparation of the HD-Ad vectors that may further improve the safety of the HD-Ad vector system (H. Zhou and A. L. Beaudet, unpublished results).

Overall, the system reported here increased the titer of vectors with E1 and E2a deletion and decreased the potential for generating replication-competent adenovirus. Since the E2a gene is essential for adenovirus DNA replication and protein synthesis, we believe that vectors deleted for both E1 and E2a will significantly improve safety when used to prepare vaccines for infectious diseases and cancer. The E1/E2a-deleted vector may also provide a safe backbone for further Ad vector development. In addition, the E2a-inducible cell lines and the E2a-deleted viruses would be useful for the study of DNA replication and virus propagation.

## MATERIALS AND METHODS

### Plasmids and viral vectors

Brief descriptions and references for plasmids and viral vectors are provided in Table 1. The pFG140 (Graham, 1984), pBHGE3, and pBHG10 plasmids (Bett *et al.,* 1994) contain adenovirus genomic DNA in plasmid forms. Plasmid pFG140 contains a small insertion of

bacterial DNA sequences in the E1 region of the Ad5 genome and is infectious in E1-complementing 293 cells. The pBHE3 and pBHG10 plasmids have a deletion of part of the E1 region, including the essential viral packaging signal. Plasmid pBHG10 also carries a deletion in the E3 region, while pBHGE3 is wild type for E3. Plasmid pΔE1spA1 is an adenovirus shuttle plasmid (Bett *et al.,* 1994). Homologous recombination of the shuttle plasmid or its derivative constructs with one of the adenovirus genomic plasmids, pBHGE3 and pBHG10, would result in the presence of infectious vector in complementing cells. The above adenovirus plasmids were kindly provided by Dr. F. Graham. Plasmid pUHD15-1 (Gossen and Bujard, 1992) and pUHD10-3 (Resnitzky *et al.,* 1994) are regulator and response plasmids, respectively, in the tTA-dependent expression system and were kindly provided by Dr. H. Bujard. Adenovirus vectors AdFG140, AdFG140ΔE2, and Ad$\beta$galΔE1E2 were constructed by the authors and reported previously (Zhou *et al.,* 1996). Other plasmids and vectors constructed in this report are also listed in Table 1 and are described elsewhere in this paper.

### Construction of E2a expression cassette and cell lines

Plasmid pUHD10-3 was first modified by cleaving its CMV core promoter with the restriction enzymes *Sma*I and *Bam*HI and then inserting a polylinker (*Sma*I–*Eco*RI–*Xba*I–*Pme*I–*Bam*HI, annealing of 5′-GGGAATTCTCTAGAGTTTAAACG and 5′-GATCCGTTTAAACTCTAGAGAATTCCC) in the deleted site. An adenovirus fragment containing the E2a ORF with its own promoters was cleaved from the plasmid pFG140 with *Eco*RI and *Dra*I and cloned into the polylinker at *Eco*RI and *Pme*I, resulting in plasmid pBZ40 (Fig. 1). *Dra*I digestion destroyed the stop codon for E2a ORF, but blunt-end ligation of *Pme*I-end 5′-GTTT and *Dra*I-end AAA-3′ regenerated the stop codon (5′-GTTT/AAA-3′). In the final construction of pBZ40, the E2 promoters are directly linked to the *tet* operators, and the E2a ORF was connected with the SV40 poly A site (Table 1 and Fig. 1). To facilitate selection of E2a-complementing cells, a neomycin resistance cassette excised with *Xho*I from pol2sneobpA (Soriano *et al.,* 1991) was inserted into pUHD15-1, resulting in pBZ47 (Table 1 and Fig. 1). Plasmids pBZ40 and pBZ47 were transfected into 293 cells by calcium phosphate precipitation (Graham, 1991). Two days after transfection, the cells were selected with 250 $\mu$g/ml G418. One of the neomycin resistance colonies (293-G7) with inducible E2a complementation was used for secondary transfection with pBZ40 and pPGKpurobpA (kindly provided by Dr. A. Bradley) to increase E2a copies in the cells. The cells were then selected with 1 $\mu$g/ml of puromycin. All cells were grown in MEM$\alpha$ (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 U of penicillin and 100 $\mu$g of streptomycin per milliliter. E2T cells were maintained in the above medium containing 10 $\mu$g/ml of tetracycline to maintain E2a expression at lower levels.

### Construction of plasmids and adenoviruses with deletion of E2a ORF

To prepare vectors defective for E1 and E2a, a segment of 1584 bp (from Ad 22445 to 24028, GenBank Accession No. M73260) covering the entire E2a ORF was first removed from a subclone of Ad sequences by using *Dra*I and *Msc*I restriction enzymes. *Msc*I cleaved at 1 bp downstream of the start codon and *Dra*I cut 1 bp into the stop codon for E2a ORF (Fig. 1B). The deletion removed all codons for the 529-amino-acid ORF for the E2a gene except the start codon. The deletion was introduced into pFG140, pBHG10, and pBHGE3 by multiple steps as described (Zhou *et al.,* 1996) and resulted in pBZ66, pBZ68, and pBZ71, respectively (Table 1). Multiple restriction sites (*Pac*I–*Xba*I–*Bst*B1–*Cla*I) were substituted at the site of the deleted E2a ORF.

Development of viral vectors from the adenoviral genomic plasmids involved transfection of adenoviral plasmids into complementing cells according to methods used for the construction of first-generation Ad vectors (Graham, 1991). AdBZ66ΔE2 was converted from plasmid pBZ66 after transfection into E2T cells.

### Vector purification, titer, and structure determinations

Plaque purification and titer determination were performed in six-well plates as described previously (Graham, 1991; Zhou *et al.,* 1996) with 293 or E2T cells. Plaques appeared about 5 to 7 days after infection of the vectors.

Two pairs of PCR primers were used for identification of E2a-deleted virus. One pair of PCR primers, 731 (5′-AGTGCGCAGATTAGGAGCGC) and 732 (5′-GCCTATAGGAGAAGGAAATG), flanking the E2a ORF, was used to analyze the WT E2a. Primer 551 (5′-CCGGCAAGTCTTGCGGCATG) located inside of the E2a ORF was used with primer 731 for detection of contamination with the WT E2a. PCR reaction conditions were 94°C for 2 min and then 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

The copy number of E2a gene was assayed by Southern hybridization of genomic DNAs cleaved with restriction enzymes, *Eco*RI and *Dra*I, cutting out the cloned E2a sequences. The control standards for copy number were prepared by adding 1, 2, or multiple microliters of $1.33 \times 10^{-5}$ $\mu$g/$\mu$l of pBZ40 into 10 $\mu$g of 293 genomic DNA, equivalent to 1, 2, or multiple copies of E2a per cell.

### Western blotting for E2a protein DBP

The treated cells were scraped off and washed in 4°C phosphate-buffered saline containing 5 mM EDTA and the cell pellet was lysed. The soluble protein in the

lysates was collected from the supernatant after centrifugation at 15,000*g* for 30 min at 4°C. After being boiled, 10-$\mu$g samples were separated on a sodium dodecyl sulfate–7.5% polyacrylamide gel and then electrophoretically transferred to a 0.45-$\mu$M nitrocellulose membrane (Bio-Rad). The membrane was washed in blocking buffer and incubated with rabbit antibody against DBP (Voelkerding and Klessig, 1986) diluted 1:2000, followed by treatment with a second antibody (horseradish peroxidase-linked anti-rabbit immunoglobulin from donkey; Amersham, Arlington, IL) diluted 1:2000. The probed DBP was detected with an enhanced chemiluminescence system (Amersham, Arlington, IL) according to the manufacturer's instructions.

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